Digest Journal of Nanomaterials and Biostructures Vol. 7, No. 4, October-December 2012, p. 1577-1586

USE OF ALKALINE LYOPHILIZATION PROCESS FOR LIGNOCELLULOSIC BIOMASS PRETREATMENT

C. A. MACARIE, A.E. SEGNEANU*, I. BALCU, R. POP, G. BURTICA[a], M. UNGUREAN[a], I. GROZESCU
National Institute of R&D for Electrochemistry and Condensed Matter - INCEMC Timisoara,
144 Aurel Paunescu Podeanu, 300569 Timişoara, Romania
[a]University „Politehnica" Timisoara, Faculty of Industrial Chemistry and Environmental Engineering, 2 Victoriei Square, 300006 Timisoara, Romania

Two pretreatment methods (alkaline lyophilization and combined microwave - acid pretreatment) were employed in order to establish their efficiency on the transformation of the lignocellulosic biomass in fermentable sugars, an important stage in the bioethanol obtaining process. Three different types of sawdust (oak, fir and hemp) were used for comparison. The enzymatic hydrolysis reaction was carried out i5n the presence of Accellerase™ 1000 enzyme complex. The amount of fermentable sugars was determined by the colorimetric method with dinitrosalicylic acid.

(Recieved August 17, 2012; Accepted October 12, 2012)

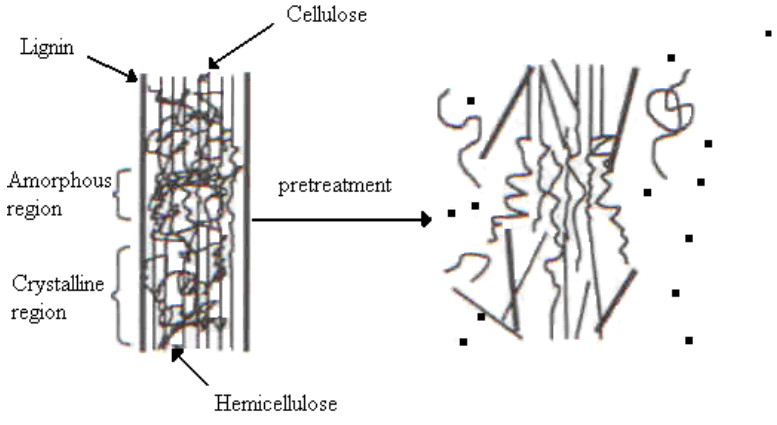
Keywords: Alkaline lyophilization, Combined microwave - acid pretreatment, Lignocellulosic biomass

1. Introduction

Among different potential alternative bioenergy resources, lignocellulosics have been identified as a prime source of biofuels and other value-added products. Lignocelluloses comprise a large fraction of municipal solid waste, crop residues, animal manures, forest residues or dedicated energy crops.

Focusing on residuals, it can be stated that lignocelluloses such as agricultural, industrial and forest residuals account for the majority of the total biomass present in the world (Kumar, 2008) and represent the most promising raw material for the biofuel production.

The first stage of the lignocelluloses conversion to ethanol consists in the biomass particle size reduction (especially through grinding) and its pretreatment, in order to destroy or remove the structural and composition possible drawbacks. The pretreatment also alters the cellulosic structure of the biomass in order to facilitate the enzymes access (Fig. 1).



*Corresponding author: s_adinaelena@yahoo.com

Fig.1. Lignocellulose structure fragmentation by biomass pretreatment

In the pretreatment stage the possible structural and composition drawbacks that may influence the hydrolysis are removed. The rate of the enzymatic hydrolysis is also improved and the yield of the fermentable sugars is increased (Mosier,2005). The pretreatment stage alters the biomass cellulosic structure in order to facilitate the enzymes access and also increases the cellulase production by the microorganisms. The pretreatment stage undertakes the breakdown of the lignocellulosic matrix in order to improve the acid or the enzymatic hydrolysis.

The major components of lignocellulosic biomass are cellulose, hemicelluloses, lignin and extractives and ash (Jorgensen,2007). The composition and ratios of these constituents depends on plant species and other factors: age plant, growth conditions, etc. (Perez, 2002), (Sun, 2002).
The composition of different lignocellulosic materials is presented in Table 1 (Kumar,2008), (Mussatto,2008).

Table 1. The composition of some lignocellulosic raw materials (as percentage based on dry weight)

Raw material	Hardwood	Softwood	Grass
Cellulose (%)	46	44	32
Hemicellulose (%)	18	22	25
Lignin (%)	26	27	18

Nowadays there are many methods used for the pretreatment of the lignocellulosic biomass: the mechanical pretreatment; self-hydrolysis or steam pretreatment; ammonia fiber explosion, which uses liquid ammonia and steam; supercritical carbon dioxide pretreatment; acid or alkaline pretreatment; ozone pretreatment; biological pretreatment that uses microorganisms (fungi) for the lignin solubilization (Champagne, 2007; Martin, 2007; Silverstein, 2007; Teymouri, 2005). Furthermore, some technological factors such as energy balance, solvent recycling and corrosion issues, as well as environmental factors such as wastewater treatment, should be carefully considered for the selected method. New applications for these kinds of fibers have been driven by increasingly more stringent environmental laws, such as the European Directives 2000/53/EC on the disposal of end-of-life vehicles and 1999/31/EC on the landfill of waste, which aim to serve the environment by enforcing waste minimization and increased levels of recycling and recovery.

The influence of the lignocellulosic biomass composition over the pretreatment stage

The cellulosic or lignocellulosic biomass is a heterogeneous complex comprising carbohydrate polymers and lignin, a complex polymer that contains monomer units of methoxylated lignols incorporated in the form of different phenylpropanoids. Unlike the starch, the cellulose structure favors the polymer chains arrangement, resulting high crystalline structures, water-insoluble and depolymerization-resistant. The hemicelluloses are branched polymers of glucose or xylose substituted with arabinose, xylose, galactose, mannose or glucuronic acid units. Lignin represents a very complex chemical compound usually derived from wood; it is also a part of the secondary cell walls of the plants. The dried mass of wood contains approximately 25-33% lignin (Chandel, 2007). As it is a very complex biopolymer, its primary structure is not accurately known.

The aim of this paper is to perform a comparative study regarding the efficiency (quantified by the yield in free glucose) of two different pretreatment methods: alkaline lyophilization and combined microwave - chemical pretreatment applied on several types of lignocellulosic biomass (herbaceous - hemp, hardwood – oak and softwood –fir sawdust). The novelty of the study is represented by the combination between lyophilisation and a chemical method applied in the pretreatment stage, as an alternative to the methods presented in the literature (Balcu, 2009; Taherzadeh, 2007).

2. Experimental

Materials and methods

All reagents were purchased from chemical suppliers and used without further purification. The enzymatic catalyst consists of Cellulase complex of Accellerase™ 1000 purchased from Genencor Accelerase™. Lignocellulosic sawdust was sieved on a digital electromagnetic sieve shaker (Filtra) and has the following dimensions: 0.85 and 1.7 mm mesh size. Lignocellulosic sawdust was milled on electrical device for lignocellulosic substrate and has the following dimension (0.8 mm mesh size).

Enzymatic hydrolysis of the sawdust without pretreatment

The hydrolysis reactions were carried on 50 cm^3 glass vessels, priorly thermostated at 40°C. Under magnetic stirring, 100 mg sawdust or cellulose standard, 10 ml acetate buffer (pH=4.8) and 50 µl enzyme Cellulase 1.5 L were added. The rate enzyme: substrate was 0.5 ml/g.

Pretreatment of sawdust

Method a – *Alkaline lyophilization pretreatment*

Suspensions (10 ml) of dry, milled sawdust (hemp, oak and fir, respectively) in NaOH 1%, were lyophilized at -52°C for 25 hours, using an Ilshin TFD8503 lyophilizer. The resulted lyophilized samples were washed with ultrapure water (25 ml), filtered and neutralized with H_2SO_4 0.82%.

Method b - *Combined microwave- acid pretreatment*

A suspension of dried sawdust (sieved or milled) and H_2SO_4 0.82% (10% dry matter, w/w) was heated in a Microwaves speedwave™MVS-2 system Berghof (P =1000 W) in the following conditions temperature: 150°C, time:10 minutes, microwave power: 500 W. The suspensions were cooled, filtered, and the sawdust was washed on the filter with distilled water and then dried in vacuum, at 60 °C.

Enzymatic hydrolysis of the pretreated sawdust

The hydrolysis reactions were carried on 50 cm^3 glass vessels, priorly thermostated at 40°C. Under magnetic stirring, 100 mg pretreated sawdust, 10 ml acetate buffer (pH=4.8) and 50 µl enzyme Cellulase complex of Accellerase™ 1000 were added. The rate enzyme: substrate was 0.5 ml/g.

The moment when the enzyme was added is considered the zero time of the hydrolysis reaction. For monitoring the evolution of the reaction, samples of 200 µl were taken at different times (during 24 hours) and the amount of total sugars was determined. The colorimetric method with 3,5-dinitrosalycilic acid (DNS) was used for the quantification of the existing sugars in the reaction mixture.

In order to calculate the total sugar content, the following Eq. (1) was used:

$$C_G = \frac{(E_p + 0.01317)10}{0.1384} \text{ [mg glucose/mL)} \qquad (1)$$

where 10 is the dilution factor, and E_p is extinction (absorbance) of the solution, measured at λ = 540 nm.

To determinate the correction factor was performed using two different blanks: pure cellulose standard (Avicel) and glucose standard. The equations used were:

$$\%_{Avicel} = \frac{291,1}{333} \cdot 100 = 87,4$$

$$\%_{glucoza} = \frac{248,7}{300} \cdot 100 = 83,0$$

In both situations, the total content of sugars from biomass was reported comparative to theoretical values (Figure 1).

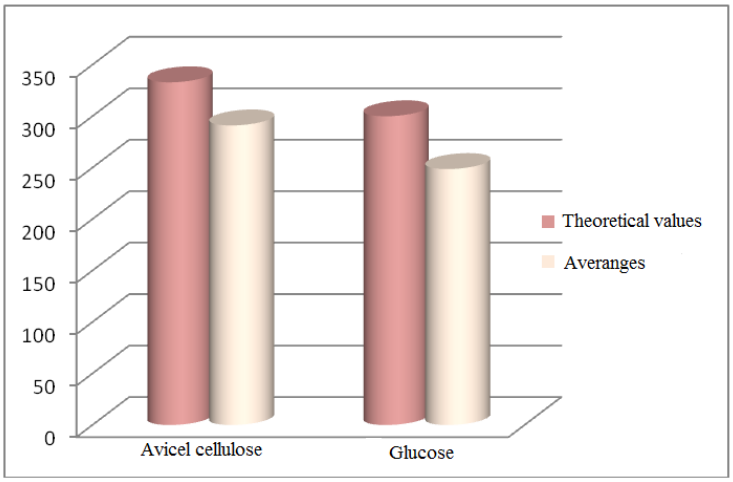


Fig. 1. Total sugar content

Considering that the average yield is 85.2 %, result that the correction factor will be 1.17.

3. Results and discussion

Experimental data

Enzymatic hydrolysis of the sawdust without pretreatment

The total sugar content, expressed as glucose, from untreated biomass samples without pretreatment, compare to Avicel cellulose was determinate after an enzymatic hydrolysis stage with cellulase (Table 2). The influences of particle size and of the biomass type were also considered.

Table2. Comparison of total sugar content from untreated lignocellulosic samples and cellulose standard.

Time (h)	Sugars content (glucose)							
	Lignocellulosic biomass substrate						Standard cellulose (Avicel)	
	Oak		Fir		Hemp			
	mg	%	mg	%	mg	%	mg	%
2	8,36	21,06	11,82	18,81	21,64	30,50	30,66	27,62
4	11,12	28,02	18,97	30,21	28,53	40,22	39,59	35,67
6	15,50	39,06	26,31	41,91	35,48	50,01	43,02	38,76
8	14,01	35,10	37,59	59,88	41,99	59,19	58,86	53,02
24	20,97	52,86	47,43	75,54	57,03	80,39	77,95	70,22

The results presented in table 1 shown that efficiency of the enzymatic hydrolysis on lignocellulosic biomass is considerably lower than on standard cellulose (Avicel). This can be explained by the fact that standard cellulose is characterized by a particle size much smaller than biomass samples, and on the other part by the fact that biomass substrate also contains lignin and other compounds inhibitors of cellulase action.

The graphical representation of Time evolution of hydrolysis reaction of various types of lyophilized biomass (Fig. 2) reveals that hemp and fir sawdusts are hydrolyzed with higher efficiency than oak sawdust.

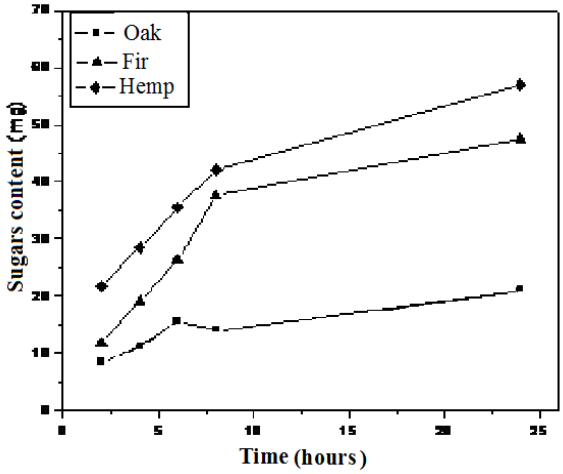


Fig. 2. Time evolution of hydrolysis reaction of various types of biomass samples without pretreatment using cellulosic complex of Celluclast 1,5L, at 40°C

Pretreatment of sawdust

Two pretreatment methods: alkaline lyophilization and microwave assisted acid pretreatment were applied to three different types of biomass: hardwood (oak), softwood (fir) and herbaceous (hemp). The pretreated biomass was subjected to enzymatic hydrolysis and the obtained amount of fermentable sugars was considered to be a possible parameter for quantifying the efficiency of the pretreatment stage.

The reducing sugar yields from enzymatic hydrolysis of alkaline lyophilized and combined microwave- acid pretreated sawdust are shown in Table 3 and 4 and Fig.3 and 4, respectively.

Table3. Results of the enzymatic hydrolysis of lyophilized sawdust used as biomass with Accellerase™ 1000 at 40°C

Time (min)	*Biomass type*		
	Hemp(with NaOH)	*Oak (with NaOH)*	*Fir (with NaOH)*
	Free glucose (μg/ mg pretreated sawdust)		
30	9.17	6.52	4.81
60	17.58	8.71	6.83
90	26.86	11.38	7.87
120	38.44	14.22	8.90
180	64.49	18.72	11.31
240	96.79	22.50	14.55
300	117.19	28.23	15.70
360	129.12	29.14	17.19
420	148.10	34.15	18.73
600	190.0	45.90	24.17
1440	227.56	69.41	40.60

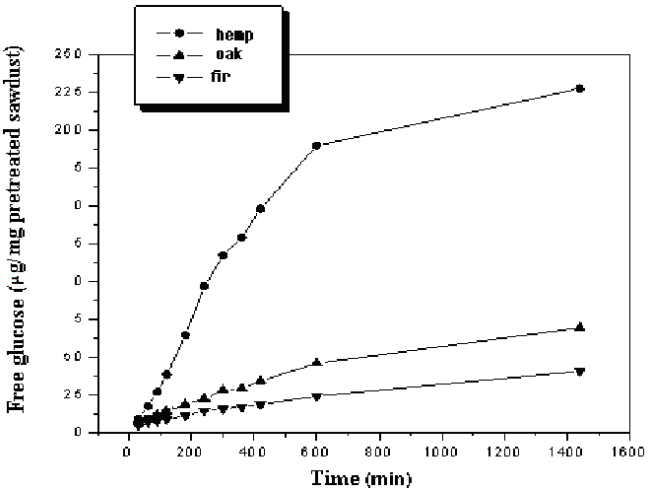


Fig. 3. Time evolution of hydrolysis reaction of various types of lyophilized biomass (with NaOH 1% at -52°C for 25 hours) using cellulosic complex of Accellerase™ 1000, at 40°C

The enzymatic hydrolysis yield depends on lignocellulosic chemical structure (Teramoto, 2008; Yang, 2006). As the above mentioned data clearly show, alkaline lyophilization pretreatment of hemp led to the obtaining of significantly higher amounts (concentrations) of reducing sugars. According to literature data, (Sun, 2002) the experimental results could be explained by the lower lignin content lignocellulosic herbaceous species. The presented data in Table 3 and Fig. 3 showed that in case of softwood (fir) the lowest values of free sugars concentration is obtained, probably due to condensation of lignocellulose (Hamelinck, 2005).

It is well known that conversion of biomass into fermentable sugars and also the bioethanol yield are influenced by the lignocellulosic substrate size (Mwaikambo, 2001). Therefore, different sized (milled and sieved) sawdust was subjected to microwave assisted acid pretreatment and then hydrolyzed, in order to establish the influence of particle size (Fig. 4 and Table 4). Also, comparisons between the two pretreatment methods will be made.

Table 4. Results of the enzymatic hydrolysis of sieved (I)/milled (II) acid pretreated sawdust with Accellerase™ 1000, at 40°C

Time (min)	***Biomass type***					
	Hemp (I)	***Oak (I)***	***Fir (I)***	***Hemp (II)***	***Oak (II)***	***Fir (II)***
	Free glucose (µg/ mg pretreated sawdust)					
30	10.89	6.22	4.33	8.0	7.80	4.52
60	15.99	7.23	6,03	9.46	7.40	4.56
90	18.83	8.18	5.25	11.73	8.48	5.25
120	21.70	8.73	5.67	12.94	9.86	5.33
180	30.48	11.04	7.33	16.88	12.71	7.57
240	43.05	18.54	8.58	20.85	17.94	8.15
300	46.06	23.06	8.15	20.98	14.60	6.76
360	61.15	19.72	12.05	17.21	19.20	11.99
420	68.47	19.45	14.46	32.71	23.60	12.10
1260	151.77	42.79	28.55	71.40	50.41	27.71
1440	149.81	42.30	30.99	63.94	62.30	32.87

I – sieved sawdust
II – milled sawdust

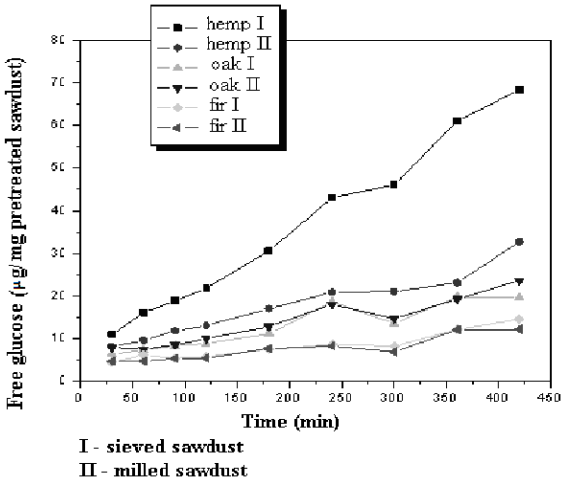


Fig. 4. Time evolution of enzymatic hydrolysis reaction with Accellerase™ 1000 of the various types of pretreated biomass with dilute sulphuric acid

The results presented in Fig. 3 and Table 3 show that best results of the enzymatic hydrolysis were obtained when sieved hemp sawdust (1.7 mm) was used.

Taking into account the results presented in Tables 3 and 4, a number of parameters (biomass type and particle size) and two pretreatment methods were analyzed in order to establish the best conditions for the biomass – bioethanol conversion process.

Influence of the sawdust pretreatment method on the yield of free sugars

As it can be seen in Fig. 5, the best results in sugars formation are obtained for the alkaline lyophilized sawdust. The acidic pretreatment of the sieved (I) and milled (II) sawdust gave poorer yields in free sugars. An increase of four to five times of the amount of free sugars (calculated as free glucose) in the enzymatic hydrolysis reaction of the lyophilized sawdust may be observed.

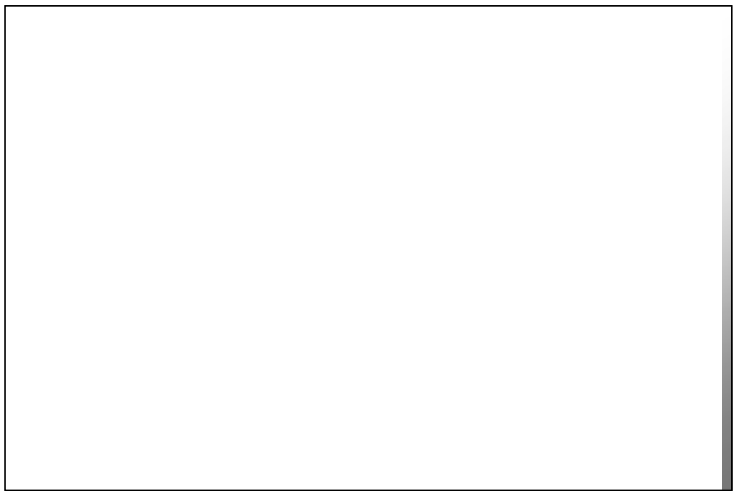


Fig. 5. Influence of the pretreatment method on the enzymatic hydrolysis of various types of sawdust

Influence of the particle size of the sawdust (sieved/milled) on the yield of free sugars

As regarding the particle size of the sawdust, the results presented in Fig. 6 show that, only in the case of oak sawdust the milling has a significant positive influence on the enzymatic hydrolysis reaction. In the case of hemp sawdust, the yield in free sugars is significantly favored by using sieved biomass than a milled one.

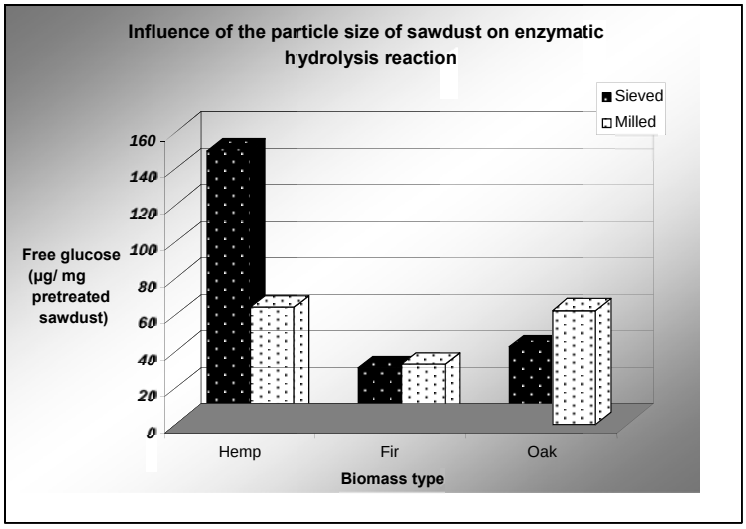


Fig. 6. Influence of the particle size of sawdust on enzymatic hydrolysis reaction

Influence of the biomass type on the yield of free sugars

As it may be seen in the Fig. 7-9, best results of the hydrolysis reaction are obtained for the hemp sawdust (Fig. 7). Pretreatment of the sawdust through lyophilization in alkaline medium and combined microwave – chemical (acid) pretreatment of the sieved hemp sawdust have led to the obtaining of good yields in free sugars.

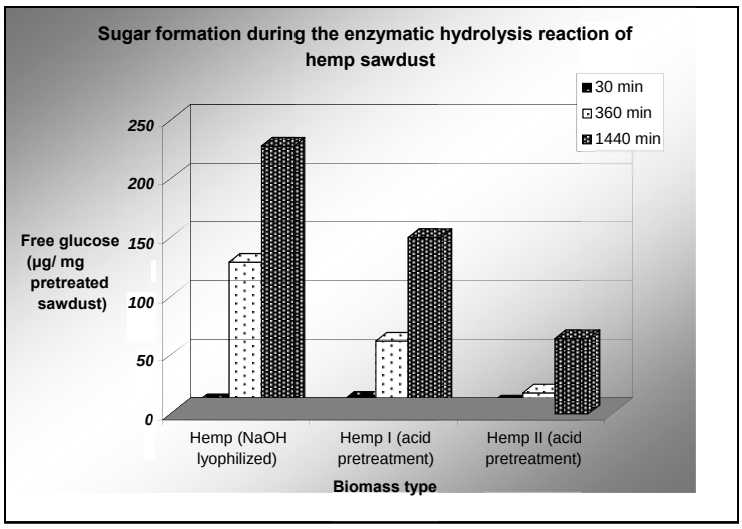


Fig. 7. Sugar formation in the enzymatic hydrolysis reaction of pretreated hemp sawdust

As regarding the oak sawdust, milling the hardwood sawdust (oak II) had a benefic influence on the evolution of the hydrolysis reaction (Fig. 8)

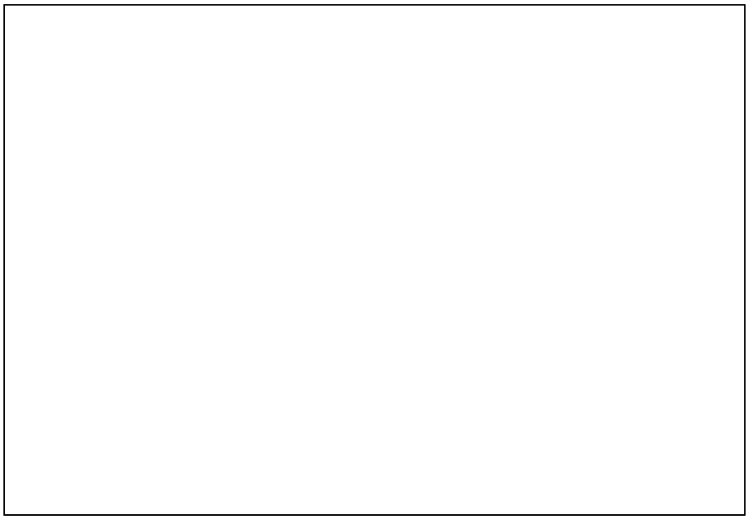


Fig. 8. Sugar formation in the hydrolysis reaction of pretreated oak sawdust

Instead, in the case of softwood sawdust (fir), there is no significantly differences between the results obtained in the enzymatic hydrolysis reaction of the sieved (I)/milled (II) sawdust (Fig. 9).

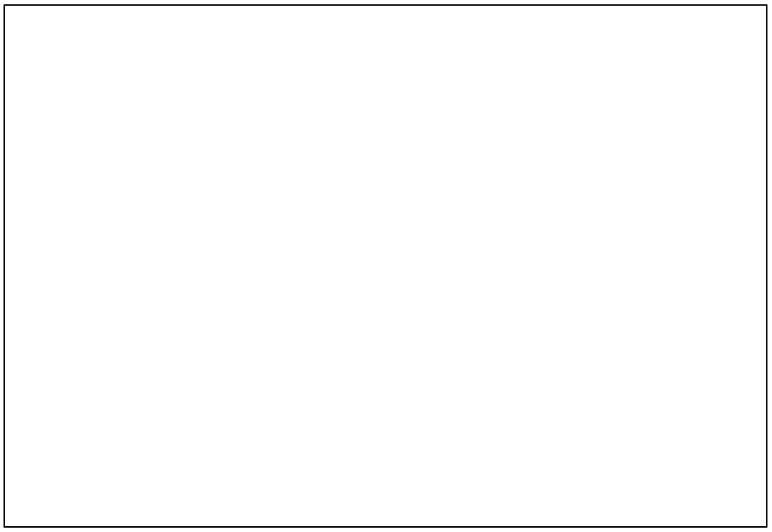


Fig. 9. Sugar formation in the enzymatic hydrolysis of pretreated fir sawdust

Best yields (expressed as free sugars concentration) of the enzymatic hydrolysis reaction are obtained when hemp sawdust is used.

The enzymatic hydrolysis results of different sawdust types show that higher glucose concentration are obtained in the case of herbaceous species (hemp) followed by the hardwood sawdust (oak). The results are explained by the level of lignin and hemicelluloses from hemp and oak which are limiting factors for enzymatic hydrolysis.

The pretreated biomass substrate contain also lignin and other inhibitors of cellulase action. It seems that lignin has no inhibitory activity itself, but adsorbs the enzyme which can't hydrolysis the cellulose under appropriate conditions.

4. Conclusions

Comparative analysis of microwave assisted acid pretreatment and alkaline lyophilization for three different lignocellulosic types in order to improve the efficiency of enzymatic hydrolysis of sawdust was performed. Based on the results discussed in this report, the following conclusions can be drawn:

Alkaline lyophilization of the biomass significantly improves the yield in free sugars and needs to be seen as a key element in biochemical conversion of lignocelluloses. Compared with

the lignocellulosic biomass conventional pretreatment methods, the alkaline lyophilization has the following advantages:

- reduces the consumption of reagents;
- pretreatment is achieved in one stage;
- simplicity and efficiency.

The results show that there is no significant improvement of the free sugars amounts when milled sawdust is used. Therefore, a mechanical pretreatment stage –sieving- is satisfactory and an energy-consumer stage (milling) can be avoided.

Regarding the biomass type, the results show that hemp sawdust gives the highest yields of free sugars.

References

[1] I. Balcu, A. E.Segneanu, M. C.Mirica, M. I.Iorga, C.Macarie, R. Martagiu, Environmental Engineering and Management Journal, **8**, 741-746, (2009).

[2] A. K. Chandel, ES Chan, R. Rudravaram, M. L. Narasu, L. Venkateswar Rao, R. Pogaku, Biotechnology and Molecular Biology Review, **2**, 14-32, (2007).

[3] P. Champagne, Feasibility of producing bio-ethanol from waste residues: a Canadian perspective, Resources, Conservation and Recycling, **50**, 211–230, (2007),.

[4] C. N.Hamelinck, G. van Hooijdonk, A.P.C. Faaij, Biomass and Bioenergy, **28**, 384–410, (2005).

[5] H.Jorgensen, J.B.Kristensen, C.Felby, Enzymatic conversion of lignocellulose into fermentable sugars: Challenges and opportunities, Biofuels, Bioproducts and Biorefining, **1**, 119–134, (2007).

[6] R. Kumar, S. Singh, O.V.Singh, Journal of Industrial Microbiology & Biotechnology, **35**, 377-391, (2008).

[7] C. Martin, B. Alriksson, A. Sjode, N.O. Nilvebrant, L.J. Jonsson, Applied Biochemistry and Biotechnology, **137–140**, 339–352, (2007).

[8] N.Mosier, C.Wyman, B.Dale, R.Elander, Y.Y.L.M.Holtzapple, M.Ladisch, Bioresource Technology, **96**, 673–686, (2005).

[9] S. I.Mussatto, M. Fernandes, A.M.F. Milagres, I. C. Roberto, Enzyme and Microbial Technology, **43** 124–129, (2008).

[10] L.Y.Mwaikambo, M. P. Ansell, Journal of Materials Science Letters, **20**, 2095-2096, (2001).

[11] J. Perez,; J. M.Dorado; T. D.Rubia; J. Martinez, Biodegradation and biological treatment of cellulose, hemicellulose and lignin: An overview, International Microbiology, **5**, 53–63, (2002).

[12] R.A. Silverstein, Chen Y., Sharma-Shivappa R.R., Boyette M.D., Osborne J., Bioresource Technology, **98**, 3000–3011, (2007).

[13] Y.Sun, J.Cheng, Bioresource Technology, **83**, 1–11, (2002).

[14] M.J. Taherzadeh, K.Karimi, Bioresources, **2**, 472-499, (2007)**.**

[15] Y.Teramoto, S.H.Lee, T.Endo, Bioresource Technology, **99**, 8856–8863, (2008).

[16] F. Teymouri, L. Laureano-Perez, H. Alizadeh, B.E. Dale, Bioresource Technology, **96**, 2014–2018, (2005).

[17] B Yang, E Wyman, Biotechnology and Bioengineering, **94**, 611-617, (2006).